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Targeting gemcitabine containing liposomes to CD44 expressing pancreatic adenocarcinoma cells causes an increase in the antitumoral activity

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Abstract

Pancreatic adenocarcinoma is often diagnosed when metastatic events have occurred. The early spread of circulating cancer cells expressing the CD44 receptor may play a crucial role in this process. In this study, we have investigated the cellular delivery ability and both in vitro and in vivo anti-tumoral activity of liposomes conjugated with two different low molecular weight hyaluronic acids (HA 4.8 kDa and HA 12 kDa), the primary ligand of CD44, and containing a lipophilic gemcitabine (GEM) pro-drug. By confocal microscopy and flow cytometry analyses, we demonstrate that the cellular uptake into a highly CD44-expressing pancreatic adenocarcinoma cell line is higher with HA-conjugated (12 kDa > 4.8 kDa) than non-conjugated liposomes. Consistently, in vitro cytotoxic assays display an increased sensitivity towards GEM containing HA-liposomes, compared to non-conjugated liposomes. Conversely, CD44 non-expressing normal cells show a similar uptake and in vitro cytotoxicity with both HA-conjugated and non-conjugated liposomes. Furthermore, we demonstrate that the HA-liposomes are taken up into the cells via lipid raft-mediated endocytosis. All the liposome formulations containing GEM show a higher antitumoral activity than free GEM in a mouse xenograft tumor model of human pancreatic adenocarcinoma. The 12 kDa HA-liposomes have the strongest efficiency, while non-conjugated liposomes and the 4.8 kDa HA-liposomes are similarly active. Taken together, our results provide a strong rationale for further development of HA-conjugated liposomes to treat pancreatic adenocarcinoma.

Highlights

► CD44-mediated uptake of hyaluronated liposomes into cells ► Lipid-raft mediated uptake of hyaluronated liposomes into CD44 expressing cells ► In vitro and in vivo inhibition of cell proliferation by hyaluronated liposomes ► Differential effects of 4.8 kDa MW or 12 kDa MW hyaluronic acid charged liposomes.

Keywords

- CD44;
- Hyaluronic acid-conjugated liposomes;
- Pancreatic cancer;
- Gemcitabine;
- Endocytosis;
- Lipid-raft

1. Introduction

Pancreatic adenocarcinoma (PDAC) is one of the most aggressive and devastating human malignancies with a death-to-incidence ratio of 0.99 and most of the patients presenting with metastatic disease at the time of diagnosis. More than 75% of patients who undergo surgical resection of small pancreatic tumors with clear surgical margins and no evidence of metastasis, die from metastasis within 5 years [1] and [2], a finding that is consistent with early spread. Recent studies

have demonstrated that in a mouse model of PDAC cellular dissemination leading to metastasis occurs prior to the formation of an identifiable primary tumor [3]. This behavior is associated with epithelial-to-mesenchymal transition and with the establishment of circulating pancreatic cells which maintain a mesenchymal phenotype and express CD44, a known feature of cancer stem cells (CSCs) [4]. Evidence for the existence of CSCs has been previously provided in primary human pancreatic adenocarcinomas grown in immunocompromised mice [5]. All of these findings suggest that CD44-targeted therapy may be a successful approach for pancreatic adenocarcinoma in association to gemcitabine (GEM), the first-line chemotherapeutic agent since 1996 [6].

CD44 is a receptor binding hyaluronic acid (HA) with an important role in CSC homing and adhesion [7]. HA binding-induced changes in CD44 membrane localization and conformation trigger the association and activation of multiple signal transduction molecules and proteases, which support migration. This biological mechanism, together with the observation that CD44 can internalize HA, indicates the possibility to recruit HA for active targeting. Using this principle, many studies have been performed with HA–drug conjugates [8], [9] and [10] or HA-targeted nanoparticles (NP) [11], [12], [13], [14], [15], [16], [17] and [18]. Of the various NPs tested, liposome-based NPs are among the best studied and clinically validated [19]. Furthermore, natural HA is a hydrophilic, non-immunogenic, biocompatible and biodegradable polymer that provides a hydrophilic shield, similar to polyethylene glycol (PEG), for the promotion of long blood circulation [20].

In the present study, we investigated the cellular delivery ability and the in vitro or in vivo anti-tumoral activity of liposomes which contained lipophilic GEM pro-drugs and were conjugated with two different MW HAs, on a highly CD44-expressing pancreatic adenocarcinoma cell line and on CD44 non-expressing normal primary pancreatic mesenchymal cells.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (HA) of different molecular weights 4.8, 12 and 51 kDa was purchased from Lifecore Biomedical (Chaska, Minnesota). All the phospholipids were provided by the Avanti Polar-Lipids distributed by Spectra 2000 (Rome, Italy). Gemcitabine (GEM, Jemta) was from Sandoz, (Varese, Italy). The pro-drug 4-(*N*)-lauroyl-gemcitabine (C12GEM) was synthesized as described by Immordino et al. [21] and was used to retain gemcitabine into the liposome formulations. Chlorpromazine hydrochloride (CPM), methyl-β-cyclodextrin (MβCD), amiloride (AMIL), and nystatin (NYS) were purchased from Sigma-Aldrich (Milan, Italy). Fluorescein-5-(and-6)-sulfonic acid trisodium salt and Lissamine™ rhodamine B-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE) were provided by Invitrogen (Life Technologies, Monza, Italy).

2.2. Cell lines

MiaPaCa2 human PDAC cell line and VIT1 normal primary pancreatic mesenchymal cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM of glutamine, and 50 μg/ml of gentamicin sulfate (Gibco, Life Technologies, Milan). Cells were incubated at 37 °C with 5% CO_2.

2.3. Liposome preparation

Liposomes composed of DPPC:Chol:PG, DPPC:Chol:PG/C12GEM or DPPC:Chol:$HA_{4.8kDa}$-DPPE/C12GEM, or DPPC:Chol:HA_{12kDa}-DPPE/C12GEM were prepared by thin lipid film hydration and extrusion method as reported [21]. Hyaluronated liposomes (HA-liposomes) were obtained adding during liposome preparation a conjugate synthesized by linking a phosphatidylethanolamine (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, DPPE) at the reducing end of HA by reductive amination. Two HA-liposome formulations were prepared containing, respectively, 4.8 and 12 kDa HA, which were fully characterized for physico-chemical properties (unpublished results). 4.8 and 12 kDa HA-liposomes had a mean diameter of 154 ± 3 and 192 ± 2 nm, respectively. The zeta potential value decreased with the increasing of the MW of the polymer and was about − 30.7 ± 0.7 mV for 4.8 kDa HA-liposomes and − 43.1 ± 0.9 for 12 kDa HA-liposomes.

2.4. Flow cytometry analysis of liposome uptake

MiaPaCa2 and VIT1 cells (3×10^5) were incubated with non-HA-liposomes, 4.8 or 12 kDa HA-liposomes labeled with fluorescein-5-(and-6)-sulfonic acid trisodium salt for different incubation times, at 37 °C or 4 °C, as indicated in the legends to figures. In competitive binding or endocytic pathway assays, MiaPaCa2 cells were pre-treated with increasing molar excess of free HA polymer (51 kDa), or increasing concentrations of CPM, MβCD, AMIL, or NYS, respectively, for 1 h, then treated with non-HA- or HA-liposomes for 1 h. The concentrations of the inhibitors, indicated in the legend in Fig. 4, were chosen in a range that was not toxic to the cells and was shown to determine a specific uptake inhibition [22], [23], [24] and [25]. Then cells were washed with PBS, trypsinized for 6 min to dissociate the surface bound liposomes, resuspended in 300 μl of PBS and analyzed on a FACSCanto dual-laser cytometer (Becton Dickinson, San Jose, CA). Flow cytometry data were gated using the FlowJo software (TreeStar, Ashland, OR). Dead cells and debris were excluded based upon forward scatter (FSC) and side scatter (SSC) measurements. Liposome uptake mediated by HA was measured by calculating the ratio between median fluorescence intensity (RMFI) of cells treated with HA-liposomes and non-HA-liposomes (fold-change).

2.5. Proliferation assays

Cells were plated in 96-well cell culture plates (4×10^3 cells/well) and incubated overnight at 37 °C with 5% CO_2. Then, cells were treated as the following: gemcitabine, C12GEM, or liposomes (DPPC:Chol:PG liposome, DPPC:Chol:PG/C12GEM liposome, DPPC:Chol:$HA_{4.8kDa}$-DPPE/C12GEM liposome, and DPPC:Chol:HA_{12kDa}-DPPE/C12GEM liposome) and further incubated for 24 or 72 h. At the end of the treatments, cell proliferation was evaluated by Crystal Violet (Sigma-Aldrich) staining and determined photometrically (A_{595} nm). Cell proliferation was reported as a percentage relative to control cells grown in the presence of an equal amount of the respective vehicle: H_2O for GEM, PBS for the liposome formulations or ethanol for C12GEM. Five independent experiments were performed for each assay condition. DPPC:Chol:PG liposomes non-containing C12GEM did not affect cell proliferation in our assay conditions (data not shown).

2.6. Confocal laser scanning microscopy

Liposome uptake has been evaluated in MiaPaCa2 and VIT1 cells. Cells were seeded in 8-chamber polystyrene vessels (1.5×10^4 cells/well) and grown overnight at 37 °C and 5% CO_2 in culture medium. Hyaluronated or non-hyaluronated liposomes labeled with fluorescein-5-(and-6)-sulfonic acid trisodium salt were added to the cells for 1 h, at 37° or 4 °C. To analyze the role of CD44 in liposome uptake, cells were also pre-incubated with 100 × molar excess of free high molecular weight HA (51 kDa) for 1 h. At the end of the incubation periods, each well was washed twice with PBS to remove the excess of vesicles, chambers were removed and cells fixed by 4% paraformaldehyde solution for 15 min. Then, each well was washed again with PBS. Cell membranes were labeled with a 25 μg/ml rhodamine-DHPE solution for 10 min to specifically highlight the cellular membranes. Then, cells were washed twice with PBS. Cover glasses were positioned by using the antifading fluorescence mounting medium (Dako). The analysis was carried out under a confocal laser scanning microscope Leica SP5 (magnification 40 × with oil immersion objective) and samples visualized using the 488 nm excitation of argon laser for fluorescein and 561 nm excitation of HeNe laser for rhodamine.

2.7. In vivo studies

MiaPaCa2 cells (4×10^6 cells/mice) were s.c. injected into the nude female mice (4 weeks of age, Harlan laboratories). One week after cell inoculation, ten randomized animals chosen for each experimental group, received solution vehicle (PBS), or 15 mg/kg of gemcitabine (GEM), or liposomes DPPC:Chol:PG/C12GEM, or liposomes DPPC:Chol:$HA_{4.8kDa}$-DPPE/C12GEM, or liposomes DPPC:Chol:HA_{12kDa}-DPPE/C12GEM by intraperitoneal injection biweekly for 4 weeks. Tumor volume and body mass were recorded biweekly for each animal. Animals were killed at the end of the 4-week study period and the tumors resected and weighted. Animal studies had been approved by the Verona University Review Board.

2.8. Statistical analysis

ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5. p values < 0.05, 0.01 or 0.001 were indicated as *, ** or ***, respectively.

3. Results

3.1. Cellular uptake of fluorescent HA-liposome formulations in PDAC cells

Several PDAC cell lines and the VIT1 normal primary pancreatic mesenchymal cells were analyzed by flow cytometry to determine the relative surface expression levels of CD44 receptor (data not shown). Among the PDAC cell lines, MiaPaCa2 showed the highest level of CD44 (485 ± 32 RMFI), while VIT1 cells did not express detectable amount of the antigen (1.18 ± 1 RMFI). These two cell types were chosen for further analyses.

Cellular uptake of HA- or non-HA-liposomes labeled with fluorescein was analyzed by flow cytometry. As shown in Fig. 1A and B, the ability of MiaPaCa2 cells to incorporate both 4.8 or 12 kDa HA-liposome formulations was significantly higher than that of VIT1 cells, suggesting that liposome uptake in MiaPaCa2 cells was mainly mediated by HA. The incubation of HA-liposomes with cells at 4 °C, which inhibits all the active energy-mediated processes, significantly reduced the uptake in MiaPaCa2 cells (Fig. 1A), suggesting that HA-liposomes entered cells via an endocytic pathway. Cells treated as described above were also analyzed by confocal laser scanner microscopy (Fig. 1C). MiaPaCa2 cells treated with fluorescein-labeled HA-liposomes for 1 h at 37 °C were strongly fluorescent. In contrast, VIT1 cells showed a very low signal, as well as MiaPaCa2 cells treated with non-targeted liposomes or at 4 °C.

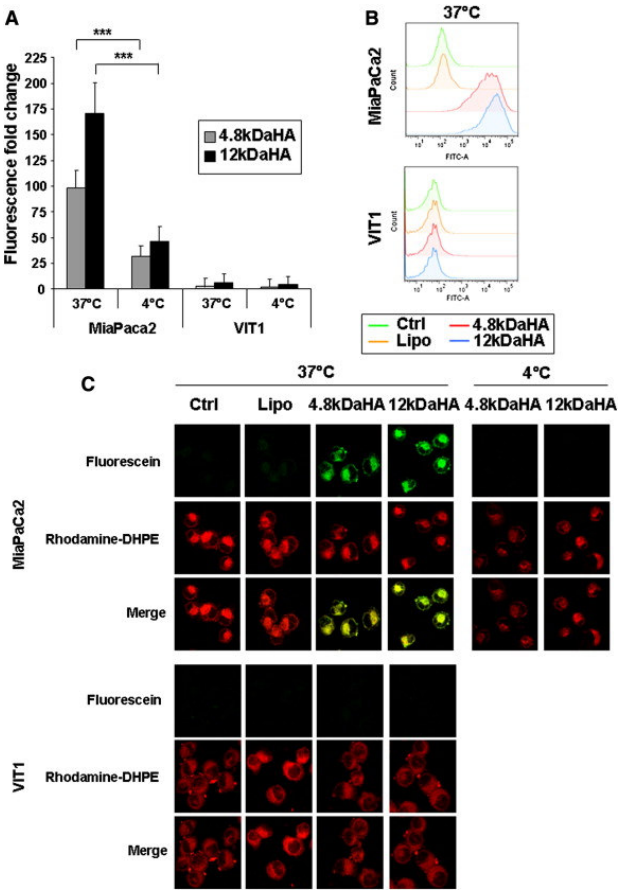

Fig. 1.

(A) Histograms of the median fluorescence intensity of MiaPaCa2 and VIT1 cells treated for 1 h at 37 °C or 4 °C, with 4.8 or 12 kDa HA-liposome formulations labeled with fluorescein. Values are the means (± SEM) of three independent experiments and are reported as the ratio between relative median fluorescence intensity (RMFI) obtained with HA- versus non-HA-liposomes (fold change). ***$p < 0.001$. (B) Representative FACS

histograms of MiaPaCa2 and VIT1 cells untreated or treated for 1 h at 37 °C with non-HA (Lipo), 4.8 kDa HA-, or 12 kDa HA-liposome formulations labeled with fluorescein. (C) Confocal microscopy images of MiaPaCa2 and VIT1 cells treated for 1 h at 37 °C or 4 °C with non-HA (Lipo), 4.8 kDa HA-, or 12 kDa HA-liposome formulations labeled with fluorescein. Green channel shows fluorescein labeled liposomes, red channel shows cellular membranes labeled with rhodamine staining, and the overlay represents cellular association of liposomes.

3.2. Influence of HA molecular weight on the cellular uptake of HA-liposomes

To evaluate the influence of HA size on the efficacy of targeted delivery, we compared the 4.8 and 12 kDa HA-liposome uptake, relative to non-HA-liposomes, at different time points. Fig. 2A shows that in MiaPaCa2 cells the specific cellular uptake occurred as early as 15 min after the beginning of the treatment with both 4.8 and 12 kDa HA-liposome formulations and reached a peak at 1 or 6 h, respectively. With longer times of incubations, up to 24 h, the presence into the cells of 4.8 kDa HA-liposomes strongly decreased, while 12 kDa HA-liposomes still remained at a high level. The HA-liposome specific uptake in VIT1 cells was not significant and did not increase with the time (Fig. 2B). However, the non-specific incorporation of liposomes in VIT1 cells, expressed as the median fluorescence intensity of cells treated with non-HA-liposomes, did increase with the time, while it remained unchanged in MiaPaCa2 cells (Fig. 2C).

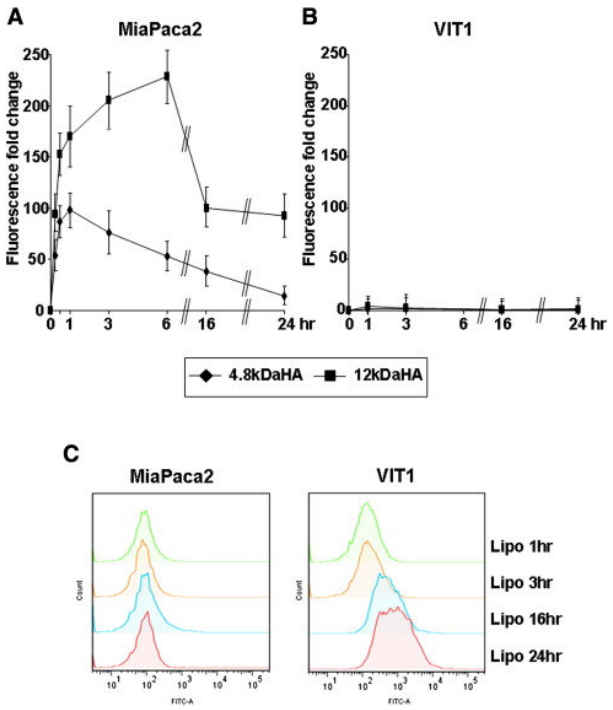

Fig. 2.

Time-dependent median fluorescence intensity of MiaPaCa2 (A) and VIT1 (B) cells treated for 15 or 30 min, 1, 3, 6, 16, or 24 h at 37 °C with 4.8 or 12 kDa HA-liposome formulations labeled with fluorescein. Values are the means of three independent experiments and are reported as the ratio between fluorescence obtained with HA- versus non-HA-liposomes. (C) Representative flow cytometry analysis of MiaPaCa2 and VIT1 cells that incorporated non-HA-liposome (Lipo) formulations labeled with fluorescein at the indicated time points. In VIT1 cells, similar results were obtained also with HA-liposomes.

3.3. Pathway of HA-liposome cellular uptake

In competitive binding experiments, pre-treatment of MiaPaCa2 cells with increasing amounts of free HA (51 kDa) significantly decreased the HA-liposome uptake (Fig. 3A and B) for both the 4.8 and

12 kDa HA-liposomes. Instead, cells treated with non-HA-liposomes did not change their fluorescence after free HA pre-treatment. In Fig. 3C, confocal microscopy analyses of MiaPaCa2 cells pre-treated with free HA before HA-liposome incubation show very weak fluorescent signals compared to HA-untreated cells. All of these findings indicate that the free ligand competes with HA-liposomes for receptor binding sites and, together with those described above, strongly support that CD44 mediates the interaction of cells with HA-liposomes.

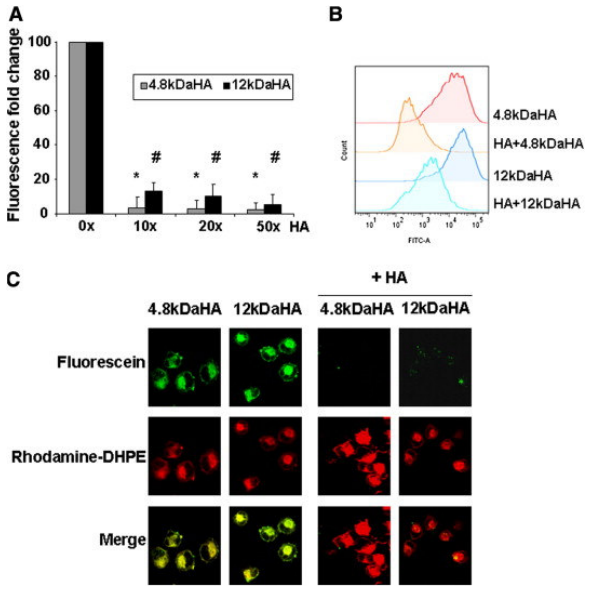

Fig. 3.

Effect of competitive ligand pre-treatment on cellular uptake of HA-liposomes in MiaPaCa2 cells. Cells were pre-incubated for 1 h with increasing molar excess of free HA (51 kDa) and treated with HA-liposomes for 1 h. (A) Histograms of the median fluorescence intensity of cells that incorporated 4.8 kDa HA- or 12 kDa HA-liposome formulations labeled with fluorescein, in the absence or presence of HA pre-treatment. Values are the means (± SEM) of three independent experiments. (*) $p < 0.001$ HA + 4.8 kDa HA vs. 4.8 kDa HA, (#) $p < 0.001$ HA + 12 kDa HA vs. 12 kDa HA. (B) Representative FACS histograms of MiaPaCa2 cells treated for 1 h at 37 °C with 4.8 kDa HA-, or 12 kDa HA-liposome formulations labeled with fluorescein, in the absence or presence of HA pre-treatment. (C) Confocal microscopy images of MiaPaCa2 cells treated for 1 h at 37 °C with 4.8 kDa HA-, or 12 kDa HA-liposome formulations labeled with fluorescein, in the absence or presence of HA pre-treatment. Green channel shows fluorescein labeled liposomes, red channel shows cellular membranes labeled with rhodamine staining, and the overlay represents cellular association of liposomes.

To determine the mechanism of the HA-liposome intracellular transport, MiaPaCa2 cells were pre-treated with increasing amounts of individual membrane entry inhibitors and then incubated with 4.8 or 12 kDa HA-liposome formulations. Flow cytometry analyses revealed that methyl-β-cyclodextrin (MβCD), an inhibitor of lipid raft formation by cholesterol depletion, strongly decreased the HA-liposome uptake, in a concentration dependent manner (Fig. 4A). In contrast, treatment with chlorpromazine (CPM), nystatin (NYS), and amiloride (AMIL), inhibitors of clathrin- or caveolae-mediated uptake or macropinocytosis, respectively, did not significantly alter the fluorescence of the cells compared to controls (Fig. 4B, C, and D). These results, together with those described above, indicate that HA-liposomes enter the cells via a lipid raft-mediated endocytosis, which depends on the binding of HA-liposomes to the CD44 membrane receptor.

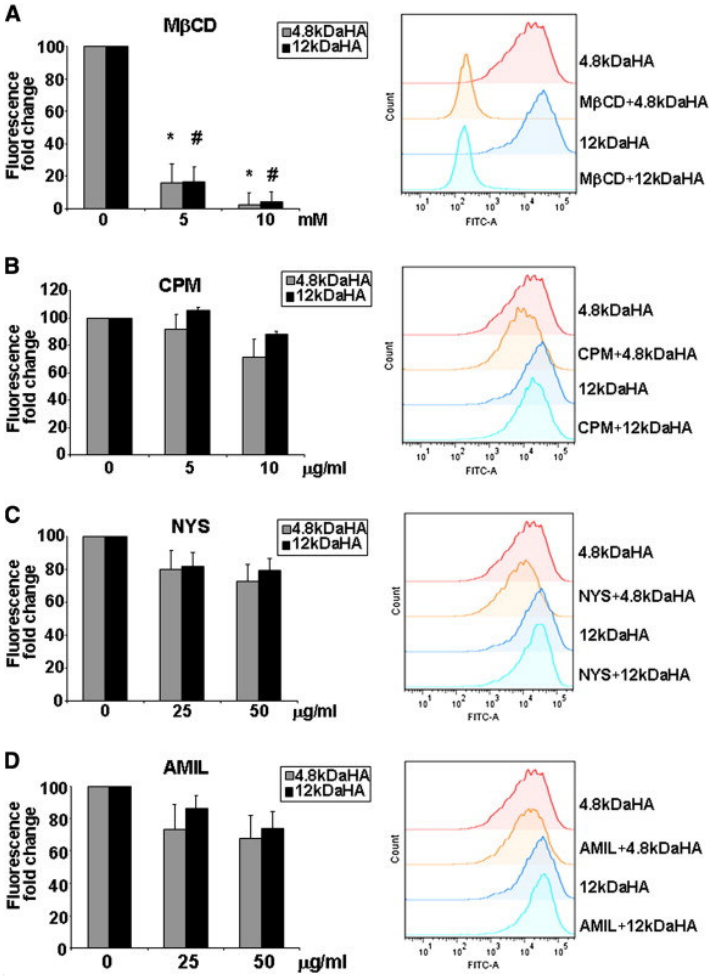

Fig. 4.

Effect of endocytosis inhibitors on cellular uptake of HA-liposomes in MiaPaCa2 cells. Cells were pre-incubated for 1 h with increasing amounts of methyl-β-cyclodextrin (MβCD) to inhibit lipid raft-mediated endocytosis (A), chlorpromazine (CPM) to inhibit clathrin-mediated endocytosis (B), nystatin (NYS) to inhibit caveolae-specific endocytosis (C), or amiloride (AMIL) to inhibit macropinocytosis (D), and treated with HA-liposome formulations for 1 h. Control experiments of cell proliferation and morphology demonstrated that CPM, MβCD, AMIL, and NYS were not toxic at all concentrations used. Figure shows the histograms of the median fluorescence intensity of cells that incorporated 4.8 kDa HA- or 12 kDa HA-liposome formulations labeled with fluorescein, in the absence or presence of endocytosis inhibitor pre-treatment. Values are the means (± SEM) of three independent experiments. (*) $p < 0.001$ endocytosis inhibitors + 4.8 kDa HA vs. 4.8 kDa HA, (#) $p < 0.001$ endocytosis inhibitors + 12 kDa HA vs. 12 kDa HA. Representative FACS histograms obtained with the highest concentrations of the inhibitors are shown on the right side of the figure.

3.4. In vitro cytotoxicity of liposome formulations against MiaPaCa2 cells

We next evaluated the cytotoxic activity of HA-liposomes containing lipophilic GEM pro-drug. As shown in Fig. 5A, all of the liposome formulations induced a concentration-dependent reduction of MiaPaCa2 cell growth up to 500 nM and were more active than free GEM or C12GEM, which displayed a similar activity. All the formulations at concentrations higher than 500 nM did not further decrease cell proliferation, consistent with our previous data obtained with GEM and various pancreatic adenocarcinoma cell lines [26]. Liposomes conjugated with HA showed a significant higher anti-proliferative activity than the non-conjugated ones and, according to flow cytometry and confocal microscopy data, 12 kDa HA-liposomes displayed a stronger effect than 4.8 kDa HA-liposomes. VIT1 cells were similarly sensitive to conjugated or non-conjugated liposomes, free GEM

or C12GEM (Fig. 5B). Cell proliferation was not significantly altered by the presence of vehicle alone in both cell lines (data not shown).

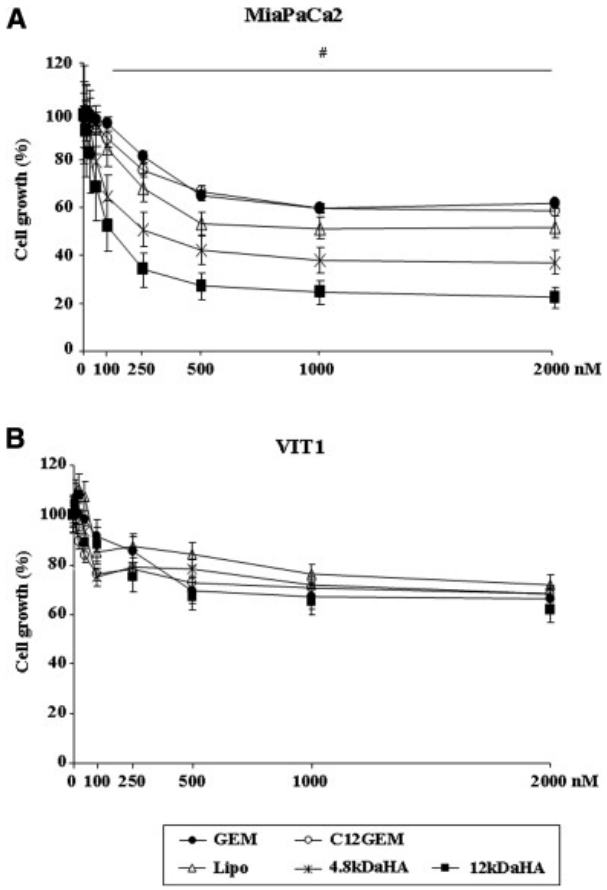

Fig. 5.

In vitro effect of liposome formulations on MiaPaCa2 (A) or VIT1 (B) cell growth. Cells were seeded in 96-well plates, incubated overnight, and treated with increasing concentrations of GEM, C12GEM, non-HA (Lipo), 4.8 kDa HA-, or 12 kDa HA-liposome formulations containing GEM pro-drug for 72 h (MiaPaCa2) or 24 h (VIT1). Cell proliferation was determined using the Crystal Violet colorimetric assay as described in the Materials and methods section. Values are the means of three independent experiments each performed in triplicate. (#) significant differences: GEM or C12GEM vs. Lipo, 4.8 kDa HA-, or 12 kDa HA-liposomes; Lipo vs. 4.8 kDa HA-, or 12 kDa HA-liposomes; 4.8 kDa HA- vs. 12 kDa HA-liposomes.

3.5. Therapeutic responses to liposome formulations in nude mice bearing MiaPaCa2 xenografts

The effect of HA-liposomes containing the lipophilic GEM pro-drug was also investigated on growth inhibition of MiaPaCa2 cells s.c. xenografted in nude mice. The examination of the volume–time curve (Fig. 6A) reveals that the volume of tumors in mice treated with 12 kDa HA-liposomes increased at a significant lower extent than that in mice treated with 4.8 kDa HA liposomes or non-targeted liposomes. During the experiment, mice body masses did not change, suggesting that the treatments did not produce any apparent toxicity (Fig. 6B). Fig. 6C shows that, at the end of the treatment period, free GEM did not inhibit the tumor mass growth in this drug-resistant pancreatic cancer xenograft model, while liposome formulations displayed different levels of efficacy. In particular, 12 kDa HA-liposomes determined a reduction in the mean tumor mass of about 50% compared to 4.8 kDa HA liposomes or non-targeted liposomes and about 65% compared to control or free GEM treatment.

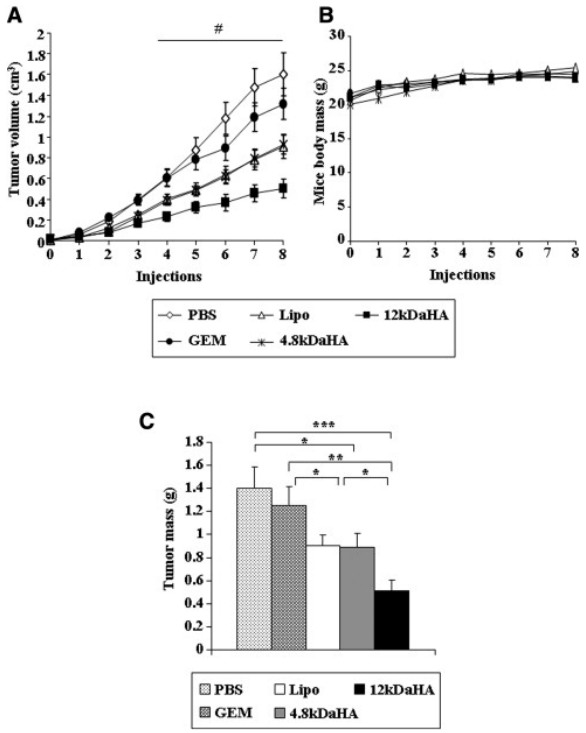

Fig. 6.

Effect of liposome formulations on xenografts of MiaPaCa2 cells in nude mice. Cells were subcutaneously injected into female nude mice. After 1 week, i.p. injections with PBS (solution vehicle), GEM, non-HA (Lipo), 4.8 kDa HA-, or 12 kDa HA-liposome formulations containing GEM pro-drug were carried out twice a week for 4 weeks, as described in the Materials and methods section. (A) Values are the means of mice tumor volume measured 3 days after each injection. (#) significant differences: PBS or GEM vs. Lipo, 4.8 kDa HA-, or 12 kDa HA-liposomes; Lipo or 4.8 kDa HA-liposomes vs. 12 kDa HA-liposomes. (B) Values are the means of mice body mass measured 3 days after each injection. (C) Values are the means of mice tumor mass (± SEM) measured after 8 injections. Significant differences: PBS or GEM vs. Lipo or 4.8 kDa HA- liposomes, Lipo or 4.8 kDa HA-liposomes vs. 12 kDa HA-liposomes, * $p < 0.05$; GEM vs. 12 kDa HA-liposomes, ** $p < 0.01$; PBS vs. 12 kDa HA-liposomes *** $p < 0.001$.

4. Discussion

We show here for the first time that a highly CD44-expressing pancreatic adenocarcinoma cell line MiaPaCa2, presents an increased uptake of liposomes carrying HA and an increased in vitro and in vivo sensitivity towards GEM containing HA-liposomes, compared to non-conjugated liposomes. Furthermore, we demonstrate that the HA-liposomes are taken up into the cells via lipid raft-mediated endocytosis, but not by clathrin- or caveolae-mediated uptake or macropinocytosis.

The HA receptor CD44 is a known surface marker of cancer stem cells (CSCs) in several human tumors, including pancreatic adenocarcinoma [4] and [5]. CD44 was first identified on lymphocytes and found to have cell adhesion and cell homing functions [27] and [28]. Thereafter, high CD44 expression was found associated with several types of malignant tumors and was shown to confer metastatic potential on non-metastasizing cell lines [29], [30], [31] and [32]. Theories on the involvement of CD44 in tumorigenesis/metastasis have been advanced and include production of autocrine growth factors by tumor cells and intracellular locomotor signals following ligand–CD44 interaction, CD44-mediated adhesion to the extracellular matrix to invade hyaluronate rich tissues, and decreased affinity for surrounding hyaluronate deficient cells leading to detachment from the primary tumor mass [7] and [32].

Recently, it has been shown that metastasis associated with pancreatic adenocarcinoma derive from the primary tumor by cellular dissemination of CSC-like cells occurring at early stages. Thus, the overexpression of CD44 on pancreatic CSCs makes HA a potentially interesting ligand for targeted therapy of pancreatic adenocarcinoma, one of the most devastating human malignancies. Targeted therapy has the advantage to minimize adverse effect by increasing drug concentration at the target site and decreasing it elsewhere in the body. This effect can be accomplished as a result of the elevated target expression on tumor cells, the long circulation of the vehiculated drug, often macromolecules or nanoparticles, and its access to the tumor better than that to normal tissues (EPR: enhanced permeability and retention effect) [33], [34] and [35]. HA exhibits a number of properties of a successful targeting ligand including its non-immunogenic nature and the presence of multiple functional groups available for chemical conjugation [36] and [37]. However, high MW HA ($> 10^3$ kDa) is normally cleared from the circulation by the hyaluronan receptor for endocytosis (HARE) on liver sinusoidal endothelial cells [38] and [39]. To reduce HA clearance from the blood, we employed short HA of 25 (4.8 kDa) and 63 (12 kDa) saccharides, respectively, taking advantage of CD44 ability to interact with a minimum HA length of 6 to 8 saccharides [40] and [41]. Selecting HA oligosaccharides long enough to bind to CD44 but too short to bind to the HARE receptor may permit an HA targeted carrier to avoid elimination by the liver while maintaining targeting to cells that overexpress CD44. Consistent with those expectations, our findings show that both 4.8 and 12 kDa HA-liposomes display high specificity towards CD44-expressing MiaPaCa2 cells, while they are poorly internalized by CD44 non-expressing VIT1 cells and at levels comparable to those of non-HA liposomes. Flow cytometry analyses performed to evaluate the kinetics of liposome uptake reveal that 12 kDa HA-liposomes are taken up by the cells at higher level than 4.8 kDa HA-liposomes, suggesting a higher affinity to CD44. This result is consistent with the data reported by Mizrahy et al. [42]. The kinetics curves also show that 12 kDa HA-liposomes remain longer into the cells than 4.8 kDa HA-liposomes. Additional experiments will be necessary to clarify the molecular mechanisms underlying the increased endocellular stability of 12 kDa compared to 4.8 kDa HA-liposomes.

It is now well accepted that liposomes are internalized into the cells through endocytosis, which can be clathrin-dependent or -independent following liposome properties, which include charge and size, cell types, and molecular composition of the cell surface [43] and [44]. Targeting liposomes to a specific receptor present at the cell surface is expected to enhance internalization activity and possibly modify the endocytic pathway relative to non-targeted liposomes. CD44 has been recently shown to use clathrin-independent endocytosis to enter cells [45]. Our confocal microscopy and flow cytometry analyses clearly show that the kinetics of incorporation of liposomes through CD44-mediated mechanisms is much faster than that occurring in the absence of CD44. However, the increase with the time of the median fluorescence intensity of the CD44 non-expressing VIT1 cells treated with all liposome formulations (Fig. 2C) suggests that non-specific liposome entrance into these cells occurs, although at a very low rate. Consistent with data reported by Qhattal et al. [23], we also demonstrate that the inhibition of lipid raft formation by cholesterol depletion completely eliminates HA-liposome internalization in MiaPaCa2 cells, while clathrin, caveolae, or macropinocytosis are not involved in the uptake process. As CD44 has been shown to localize in cholesterol-rich lipid raft [46], which are responsible for its stability [47], we may argue that cholesterol depletion inhibits HA-liposome cellular uptake by influencing CD44 stability.

GEM (2′,2′-difluoro-2′-deoxycytidine, dFdC) is a nucleoside analogue of deoxycytidine used as a first line therapy for pancreatic adenocarcinoma [6]. Most administered GEM undergoes rapid metabolism to dFdU by deamination primarily in the blood, making GEM half-life as short as 8–17 min in human plasma [48] and [49] and 9 min in murine plasma [50]. We have previously synthesized

and encapsulated 4-(N)-acyl-GEM derivatives into liposomes and demonstrated its longer plasma half-life and higher in vitro cytotoxicity compared to the free drug [21]. Furthermore, we have demonstrated that HA-lipoplexes are efficiently transfected into CD44-expressing breast cancer cells [51]. Here, we show that HA-targeted liposome formulations containing GEM have an in vitro cytotoxic activity against CD44-expressing pancreatic adenocarcinoma cells higher than that of non-targeted liposomes or free GEM. In contrast, CD44 non-expressing cells were similarly sensitive to non-targeted or targeted liposomes. These findings are consistent with those obtained in flow cytometry and confocal microscopy analyses.

Our in vivo results obtained with a mouse xenograft tumor model of human pancreatic adenocarcinoma show that all the liposome formulations containing GEM were more efficient than free GEM in inhibiting MiaPaCa2 cell growth, which mirror the results obtained in the in vitro assay. However, while the 12 kDa HA-liposomes showed the strongest antitumoral activity, non-HA-liposomes and the 4.8 kDa HA-liposomes had a similar efficiency. To interpret this finding, we should point out that non-HA-liposomes are not subjected to the clearance from the blood via receptor-mediated mechanisms, such as HARE. We may postulate that this advantage of non-HA-liposomes could counterbalance the higher binding affinity of HA-liposomes, therefore rendering them, in an in vivo assay, as active as the 4.8 kDa HA-liposomes, but still less active than the 12 kDa HA-liposomes. Our report describes for the first time in vivo experiments performed using low MW HA-liposomes. Peer and Margalit [17] and [18] have reported that liposomes charged with high MW HA and containing doxorubicin or mitomycin C increase the antitumoral activity of the non-targeted drug in various mice tumor models.

Taken together, our results provide a strong rationale for further development of HA-conjugated liposomes, containing single or combined drugs, to be used for the treatment of pancreatic adenocarcinoma. Future studies will be addressed to evaluate the maximum tolerable dose (MTD) for the liposomal formulations of GEM in order to evaluate their therapeutic potential and the possible side effects.

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